Mobile Actin Clusters and Traveling Waves in Cells Recovering from Actin Depolymerization

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ABSTRACT At the leading edge of a motile cell, actin polymerizes in close apposition to the plasma membrane. Here we ask how the machinery for force generation at a leading edge is established de novo after the global depolymerization of actin. The depolymerization is accomplished by latrunculin A, and the reorganization of actin upon removal of the drug is visualized in Dictyostelium cells by total internal reflection fluorescence microscopy. The actin filament system is reorganized in three steps. First, F-actin assembles into globular complexes that move along the bottom surface of the cells at velocities up to 10 μm/min. These clusters are transient structures that eventually disassemble, fuse, or divide. In a second step, clusters merge into a contiguous zone at the cell border that spreads and gives rise to actin waves traveling on a planar membrane. Finally, normal cell shape and motility are resumed. These data show that the initiation of actin polymerization is separated in a contiguous zone at the cell border that spreads and gives rise to actin waves traveling on a planar membrane. Finally, normal cell shape and motility are resumed. These data show that the initiation of actin polymerization is separated in a contiguous zone at the cell border that spreads and gives rise to actin waves traveling on a planar membrane.

INTRODUCTION

Lamellipodia are the driving organelles in actin-based cell motility (Small et al., 2002). They consist of a dense network of actin filaments which abuts upon the plasma membrane in a zone known as a leading edge. The force for pushing the leading edge forward is thought to be provided by the polymerization of actin, and details of the machinery that couples this process to the membrane are under discussion (Dickinson et al., 2002; Mogilner and Oster, 2003). Actin assembles at the membrane into a dense network, together with proteins that regulate the branching, cross-linkage, and membrane anchorage of the actin filaments. In contact with a substratum, the forces generated by the polymerization of actin are translated into forward propulsion of the entire cell (Pollard and Borisy, 2003).

The question addressed in this work is how the machinery for force generation is reassembled within a cell after the global depolymerization of actin. In Dictyostelium cells moving on a glass surface, the cortical actin system is differentiated into a loose network of single or bundled filaments and into dense assemblies. Dense assemblies of filamentous actin constitute not only the leading edge; they are also generated beneath the substrate-attached cell surface in the form of short-lived foci and traveling waves (Bretschneider et al., 2004). These actin-based structures are completely destroyed by treating the cells with latrunculin A, an inhibitor of actin polymerization (Spector et al., 1989; Pring et al., 2002) that binds in a cleft between subdomains II and IV of monomeric actin (Morton et al., 2000). Latrunculin A acts by sequestering monomeric actin (Yarmola et al., 2000; Morton et al., 2000). As a consequence, filamentous actin structures fall apart in living cells depending on the rate of actin depolymerization. After the removal of latrunculin A, Dictyostelium cells recover from the treatment, and the reconstitution de novo of leading edges can be recorded.

There are, in principle, two ways in which actin polymerization is recovered after complete depolymerization: 1), polymerization is regained in situ, meaning at the border of the cell where leading edges had been formed before latrunculin A was added; or 2), only at a second step the polymerization is linked to the sites where protrusion is going to occur. Here we provide evidence for this second pathway of actin repolymerization and leading-edge reconstitution in Dictyostelium. As a technique to recognize details of actin organization we use total internal reflection fluorescence (TIRF) microscopy in combination with GFP-tagged constructs of LimE, a LIM domain protein of Dictyostelium (Schneider et al., 2003). By fluorescence as...
by electron microscopy, a construct lacking the C-terminal coiled-coil domain of the LimE protein (LimE\textsubscript{Dcoil}) turned out to be the best label for the cortical actin filament network in \textit{Dictyostelium} cells (Bretschneider et al., 2004). The application of LimE\textsubscript{Dcoil}-GFP to TIRF microscopy proved to be optimal for distinguishing in live cells the actin-filament system from the background of unpolymerized actin. Under the optical conditions used, fluorophores deeper than 200 nm within a cell are no longer detected, which sets a limit for the contribution of background fluorescence from the cytoplasm.

**MATERIALS AND METHODS**

**Culture and treatment of \textit{Dictyostelium} cells with latrunculin A and FM4-64**

Various cell lines derived from \textit{D. discoideum} strain AX2-214, expressing GFP- or mRFP-tagged LimE constructs, GFP-ABD120 (Pang et al., 1998), or GFP-actin (Westphal et al., 1997) as probes, were used in this study. LimE-null cells were complemented with full-length LimE-GFP (see Figs. 3 and 4) or with truncated LimE\textsubscript{Dcoil} that lacks its C-terminal coiled-coil domain and carries GFP at its C-terminus (see Figs. 1 and 2; Figs. 5–7 and 10; and Movies 2 and 3). Wild-type AX2-214 cells were transformed with a pDEX vector to express LimE\textsubscript{Dcoil} carrying either GFP or mRFP at its N-terminus (see Figs. 8 and 9, and Movies 1 and 4). Disruption of the \textit{DdLimE} gene and generation of the GFP constructs were described by Schneider et al. (2003). Cells were cultivated in nutrient medium in polystyrene culture dishes at 23 ± 2°C; ~1 h before an experiment, cells were washed twice in 17 mM K/Na-phosphate buffer, pH 6.0 (in this article referred to as “phosphate buffer”).

Latrunculin A, fluorescent dextrans, and FM4-64 were purchased from Molecular Probes (Eugene, OR). A stock solution of 1 mM latrunculin A in dimethylsulfoxide was diluted 100-fold in phosphate buffer and mixed 1:1 with the buffer on top of the cell layer, yielding a final concentration of 5 \( \mu \text{M} \). For full recovery, the cells were washed and incubated with phosphate buffer, and the washing was repeated after ~40 min. Texas Red dextran (3000 mol wt, neutral) was applied at a final concentration of 2 mg/ml, and Alexa-Fluor 488 dextran (10,000 mol wt) at 0.5 mg/ml in phosphate buffer. Since cells exposed to the fluorescent dextrans were extremely light-sensitive and tended to contract, dithiothreitol was added to a final concentration of 20 mM to record the long time series of wave propagation shown in Fig. 8 and Movie 4. For FM4-64 labeling, a stock solution of 1 mg/ml in DMSO was diluted in phosphate buffer to a final concentration of \( \frac{1}{3} \times 10^{-2} \) \( \mu \text{g/ml} \) for TIRF microscopy or 1 \( \mu \text{g/ml} \) for confocal scanning.

For actin determination, proteins were separated by SDS-Page in 10% minigels and stained with Coomassie BlueR. The gels were scanned using a ScanJet 5370C (Hewlett-Packard, Palo Alto, CA). After subtraction of background the actin bands were analyzed with the program AIDA Version 2.11 (Raytest Isotopenmessgeräte, 45549 Sprockhövel, Germany).

**FIGURE 1** Actin depolymerization by latrunculin A, and characteristic patterns of reorganization after removal of the drug by washing in phosphate buffer. The images represent confocal scans through cells of \textit{Dictyostelium discoideum} at planes close to the substrate-attached cell surface. Structures labeled with three different probes are compared. (A) GFP-actin; (B) GFP-ABD120; and (C) LimE\textsubscript{Dcoil}-GFP. The panels show, from left to right, cells moving on a glass surface before the treatment with latrunculin A, showing leading-edge labeling with all three probes and actin network structures in B and C; cells after 16–20 min of incubation with 5 \( \mu \text{M} \) latrunculin A, in which the labels are uniformly distributed in the cytoplasm and only organelles are spared; patches are the first structures recognized after the wash-out of latrunculin A; waves are typical of a later stage of reorganization before normal cell shape is recovered. Patches are formed within the first 15 min after the removal of latrunculin A, waves are most abundant after 20 to 30 min, and recovered cells are observed after 40 min or longer. For A and B, probes were expressed in a wild-type background, for C the probe was expressed in LimE-null cells. Bar, 10 \( \mu \text{m} \).
TIRF and confocal microscopy

For live cell imaging, Plexiglas rings (20 mm diameter, 4 mm height) were mounted with silicone vacuum grease or paraffin onto glass coverslips. Cells were allowed to settle on these coverslips. For through-the-objective TIRF standard coverslips and conventional immersion oil were used. Most of the single-color images were acquired on a Zeiss Axiovert 200 M microscope equipped with an α-Plan-Fluar 100×/1.45 NA oil immersion objective in conjunction with a Till Photonics (82166, Graefeltting, Germany) TIRF condenser and a bandpass GFP filter set (excitation 488/10, DC 498; emission 515/30, Chroma Technologies, Rockingham, VT). An Optovar 1.6× was used to enlarge the image on the camera chip. Fig. 3 B was acquired using a Nikon TE2000 inverted microscope, equipped with the Nikon TIRFM module, a 100×/1.45 NA oil immersion objective and a modified Zeiss filter set 09 (excitation 488/10, FT 510, emission 520 LP).

Simultaneous dual color TIRF (see Figs. 8 and 9, and Movies 1 and 4) was performed using an Olympus IX-70 microscope, a 60×/1.4 NA Plan-Apo objective, and a Till Photonics condensor. A 488/10 filter was used for excitation of both GFP and FM4-64. Red and green fluorescence signals were split using a Multi-Spec dual emission splitter (Optical Insights, Santa Fe, NM) having a 595 nm dichroic for the separation of green and red emissions, which were further passed through 510–565 and 605–655 nm bandpass filters, respectively.

In all three systems, the 488-nm line from an Argon/Krypton ion laser (Innova 90, Coherent, Santa Clara, CA) coupled into an optical single-mode fiber (OZ Optics, Carp, Ontario, Canada) was used for illumination. Images were acquired by a frame-transfer, back-illuminated 16-bit CCD camera (MicroMax 512BFT, pixel size 13 μm, Roper Scientific, Trenton, NJ) using the Metamorph Imaging System (Universal Imaging, 82178, Puchheim, Germany). Laser power was optimized such that sufficient contrast was achieved with minimized optical impact on the cells.

Confocal scans of live cells were obtained using a Perkin Elmer Ultra View (Beaconsfield, UK) spinning disc system on a Nikon Eclipse TE300 inverted microscope, equipped with a 100×/1.4 oil Planapo objective and a Hamamatsu ORCA ER camera model C4742-95-12ERG. For the images shown in Fig. 1, the exposure time was 100 ms; GFP fluorescence was excited at 488 nm, and a 500 nm long-pass filter was used for emission. For Fig. 10, stacks of images were obtained with a step size of 500 nm in the z direction. GFP and FM4-64 were both excited at 488 nm, and the emissions separated by 525 and 700 nm bandpass filters. Green and red emissions were recorded alternately with an exposure time of 50 ms for each channel.

For phallolidin and immunolabeling (Fig. 2), LimE-null cells expressing LimEcoil-GFP were fixed for 15 min with picric acid/formaldehyde followed by 70% ethanol according to Humel and Biegelmann (1992). The cells were labeled with TRITC-phallolidin (0.5 μg/ml, Sigma, St. Louis, MO), and subsequently with affinity-purified rabbit anti-phallolidin antibody followed by Alexa Fluor 488-conjugated goat anti-rabbit antibody (2 μg/ml, Molecular Probes). Confocal fluorescence images were obtained on a Zeiss LSM 410 equipped with a 100×/1.3 oil Plan-Neofluar objective. For TRITC, excitation was at 543 nm and a 590–610 nm filter was used for emission. For Alexa Fluor 488, excitation was at 488 nm and emission was recorded at 510–525 nm.

Line scan analyses (see Fig. 3, and Figs. 7 and 8), tracking of patches (see Fig. 6), and superposition of red/green pseudo-colored image sequences (see Fig. 7, and Figs. 8 and 9) were performed using ImageJ software (http://rsb.info.nih.gov/ij). In Fig. 7, trajectories of cell fronts were traced manually in the binarized time-space plot (see Fig. 7 C) and imported to Microsoft Excel for computing the increase of the cell diameter in time (see Fig. 7 D). Differentiation of the cell diameter with respect to time, and division by 2 gives the averaged velocity of front expansion (see Fig. 7 E).

RESULTS

Actin depolymerization and reassembly monitored in live cells

In search for an optimal fluorescent label to monitor the depolymerization of actin and its reorganization after the treatment of cells with latrunculin A, we have compared three GFP-tagged probes: N-terminally tagged actin, the actin-binding domain of a 120-kDa filamin homolog of Dictyostelium (ABD120), and a truncated version of the Lim-domain containing protein LimE (LimEΔcoil). The calponin-like ABD120 has a molecular mass of 28 kDa, and the truncated LimE, which lacks the coiled-coil region at the C-terminus of the polypeptide sequence, has a molecular mass of 15 kDa. These probes were previously used to study actin network organization in untreated, motile Dictyostelium cells (Bretschneider et al., 2004).

All three probes showed the complete loss of any microscopically detectable actin structure within 10–20 min of treatment with 5 μM latrunculin A. During recovery after wash-out of the drug, these probes revealed two consecutive patterns of dense actin assemblies: first the appearance of separate patches, and subsequently wave patterns associated with the substrate-attached membrane and the lateral cell border (Fig. 1); ~1 h after the removal of latrunculin A, normal cell shape and motility was restored.

The three fluorescent probes resembled each other in the overall pattern they recognized. However, in terms of optimal resolution of delicate actin structures, GFP-actin was inferior to the two other probes because of a high background produced by unpolymerized actin in the cytoplasm. The cytoplasmic background proved to be slightly lower with the LimEΔcoil probe than with ABD120, in accord with our previous observations on untreated cells (Bretschneider et al., 2004).

To establish that live-cell imaging using LimEΔcoil-GFP as a probe parallels the labeling of filamentous actin in fixed cells using fluorescent phallloidin, we have superimposed...
the two labels on top of each other. Fig. 2 A shows a cell expressing LimEΔcoil-GFP, fixed during the recovery from latrunculin A treatment. The cell was double-labeled with red fluorescent phalloidin (Fig. 2 B) and with anti-GFP antibody followed by green fluorescent Alexa Fluor 488-conjugated second antibody (Fig. 2 C). Merging of the two labels is indicated by yellow color (Fig. 2 D). In conclusion, the same type of structures is recognized during the depolymerization of actin by four different probes: directly GFP-tagged actin, the calponin-like actin binding domain ABD120, a Lim-protein derived construct, and phalloidin.

Since delicate actin network structures in the cortex of Dictyostelium cells are better resolved by TIRF than by confocal microscopy, we show consistently TIRF images in the following analysis of actin dynamics before and after the treatment of cells with latrunculin A. We will finally turn back to confocal scans for three-dimensional reconstructions of the actin patterns.

**Leading-edge dynamics in motile Dictyostelium cells**

As a reference for stages of actin reorganization during the recovery from latrunculin A, we illustrate in Fig. 3 the dynamics of actin assembly in untreated Dictyostelium cells. In these motile cells, lamellipodia of various shape are continually extended in different directions. The dynamics of leading edge protrusion and retraction can be studied by TIRF microscopy as long as the front of a cell is in close contact with the substrate (Fig. 3 A). To ensure that the very front is captured by the evanescent illumination, we show in Fig. 3 B a cell that partially spreads underneath another one. The fact that actin networks in both cells can be seen provides evidence that, in this example, the front of the undercutting cell (to the lower right in Fig. 3 B) is illuminated in its entire depth. In this cell, as in the one shown in Fig. 3 A, the intensity of the actin label sharply peaked in the middle of a zone nearly 1 μm in width (Fig. 3 C). These images indicate that the specific actin organization at an advancing cell edge is restricted to a narrow zone wherein the packing density of actin filaments reaches a peak of ~5-fold the average density in the trailing loose network. Temporal changes in fluorescence intensity, measured at a position stationary relative to the substrate, show that the passage of a leading edge creates a sharp peak with a width at half-maximum of 9 s, indicating that the assembly of actin at the leading edge is promptly followed by disassembly (Fig. 3 D).

**Pathway of actin depolymerization by latrunculin A**

Within less than half a minute of latrunculin A treatment, the filamentous network in the cortex of Dictyostelium cells breaks down into patches, which are mobile, and the dense accumulation of F-actin at leading edges falls apart into separate clusters (Fig. 4 A). Simultaneously, the actin bundles within filopodia disappear, followed by retraction of these surface extensions. The rapid disruption of the network (second frame of Fig. 4 A) illustrates that maintenance of normal actin organization in the cell cortex requires continuous cycling between the polymerized and un polymerized state of actin.

Despite the presence of latrunculin A, patches of dense actin assembly appeared de novo at the substrate-attached surface and at the border of the cells. This means that global depolymerization was locally counteracted by the appearance of actin-rich clusters (arrowheads in the 99-s frame of Fig. 4 A and Movie 1). These deviations from a monotonic progression of actin disassembly suggest that nucleation is not completely inhibited at intermediate stages of latrunculin A action.
The final stage of actin depolymerization led to membrane pearling, as previously observed in a fibroblast cell line and explained as an instability phenomenon (Bar-Ziv et al., 1999). The instability occurs in a membrane tube when the shear rigidity in the actin cortex is reduced. At that stage the cell is surrounded by protrusions of the plasma membrane, which consist of spherical expansions spaced by thin tubular connections (Fig. 4A, 236-s frame, and Fig. 4B). Combination of FM4-64 to label the plasma membrane with LimE-coil-GFP to label actin assemblies during latrunculin A treatment revealed a connection of pearling to the site of actin clusters retained at the membrane before the final stage of complete depolymerization (Movie 1). As a result, pearls are often enriched in the fluorescent LimE-coil label relative to the cytoplasm.

Sequence of actin patterns formed during the recovery from latrunculin A

The complete disappearance of F-actin assemblies in cells treated with latrunculin A provides an excellent starting condition for a step-by-step investigation of the reconstitution of the apparatus responsible for cell motility. The question is whether nucleation sites are retained at the cell border, or if actin repolymerization can be initiated anywhere on the cell surface and subsequently linked to protrusion at a leading edge.

The first phase of actin repolymerization after latrunculin A wash-out was the assembly of dense actin patches irregularly distributed over the bottom surface of the cells. Patches appeared also along the pearled surface extensions of the cells (Fig. 5, A and B). In a second phase, the central region of the bottom surface became depleted of actin clusters, whereas patches accumulated near the border of the cells. These clusters grew in size and fused into a contiguous zone, thus conferring the activity of a protruding front to the cell border (Fig. 5C). The third and penultimate period of recovery was characterized by the profuse formation of concentric or spiral-shaped actin waves all over the ventral cell surface (Fig. 5D). Eventually, this wave activity declined (Fig. 5E) and normal actin network organization, cell shape, and leading-edge activity recovered (Fig. 5F).

The recovery of normal actin organization and cell movement after the wash-out of latrunculin A takes long enough for the rates of actin synthesis or degradation to be altered. To examine whether the sequence of actin patterns observed during recovery results from an up- or down-regulation of total cellular actin, we have determined actin in parallel to the formation of wave patterns. In Coomassie-Blue stained SDS-gels we did not find any significant net change in total actin during an incubation period of 10 min with 5 μM latrunculin A and within 30 min thereafter in phosphate buffer, which comprised the period of extensive wave formation (results not shown). The total amount of cellular actin varied during the experiment only within the limits of ±15%, suggesting that pattern formation is regulated at the posttranslational level.

The turn from patch movement to spreading fronts and wave dynamics

The globular actin clusters formed during the first phase of recovery slid along the ventral cell surface, able to divide and fuse with other clusters. Their velocities varied from 0 to 11 μm × min⁻¹, with no preferential orientation of trajectories (Fig. 6). Once formed, these patches acted as transitory centers of actin polymerization; they grew in size and faded

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**FIGURE 4** Disassembly of F-actin structures and membrane “pearling” induced by latrunculin A. The fluorescent label is LimE-GFP in LimE-null cells. (A) Time course of the disappearance of actin structures during the incubation of a cell with 5 μM latrunculin A. Time is indicated in seconds before (first frame) and after addition of the drug (following frames). Arrowheads in the 99-s frame point to dense actin assemblies that are transiently formed against the trend of overall depolymerization. (B) A cell incubated for 10 min with 5 μM latrunculin A, showing intense pearling in extensions of the cell surface. Bars, 5 μm in A; 10 μm in B.
out independently of each other, indicating that the persistence of each patch depended on an intrinsic balance of actin polymerization and depolymerization.

The most remarkable events in recovery from latrunculin A are 1), the transition from the motility of independent actin clusters to the coordinated protrusion of a leading edge; and 2), the excessive formation of travelling waves. A sequence of events is illustrated in Fig. 7 and in the supplemental Movies 2 and 3. Once established as a contiguous zone along the cell border, the front spread with a speed of $2 \mu m \times min^{-1}$. In a next step, the contiguous zone broke into concentric or spiral-shaped waves that travelled on the bottom surface of the cells. The phase of wave formation could be prolonged by diluting latrunculin A from the inhibitory concentration of 5 $\mu M$ down to 1 $\mu M$, rather than completely removing the drug (Movie 2).

**Is wave propagation coupled to membrane protrusion?**

The question of whether the waves formed during recovery from actin depolymerization travel freely on a planar membrane or in connection with a membrane fold, has been addressed by double labeling, showing the profile of the bottom surface in parallel to wave propagation. There is a choice of two TIRF methods to visualize the profile of a cell surface in contact with a substrate (Axelrod, 2003): 1), to incorporate a fluorescent dye into the plasma membrane (Axelrod, 1981); and 2), to allow an impermeant macromolecule, for instance fluorescent dextran, to diffuse into the liquid space between the cell and substrate surfaces (Gingell et al., 1985). The latter technique has been introduced by Todd et al. (1988) to overcome the ambiguity of interference.
reflection microscopy in mapping cell-glass contacts. We have applied both TIRF methods to probe for protrusions of the cell surface beneath or in front of a propagating actin wave. Using the second method, similar results were obtained by combining LimEΔcoil-GFP with Texas Red dextran (3000 mol wt), or red mRFP-LimEΔcoil label for actin with green fluorescent Alexa-Fluor 488 dextran (10,000 mol wt). No change in proximity of the cell surface to the substrate was detectable when a wave traveled over a membrane area (Fig. 8 and Movie 4). The wave pattern shown in Fig. 8 illustrates a characteristic feature observed upon collision of two wave fronts: the waves do not cross each other but fuse laterally, giving rise to wave propagation perpendicular to the initial direction.

Alternatively, cell membranes were labeled by the red fluorescent dye FM4-64, which can be excited with the same 488-nm laser line as GFP. Superposition of the actin and membrane labels confirmed that the actin waves can spread on a planar membrane with no leading edge or membrane fold in front of them (Fig. 9). However, when a wave reached the cell border, it was capable of pushing this barrier forward (Fig. 9, 29- to 105-s frames), demonstrating that wave propagation can be facultatively coupled to leading-edge protrusion.

Three-dimensional organization of actin patches and waves

TIRF microscopy as used in this article reveals high-resolution images of actin-filament structures in the plane of the substrate-attached cell surface. However, TIRF does not provide information on the structure of actin complexes perpendicular to the cell surface. To relate the actin assemblies formed at the bottom surface of the cells to the shape of the cell body, we have scanned cells recovering from actin depolymerization in the z direction using a spinning-disk confocal microscope. Cells expressing LimEΔcoil-GFP to visualize actin structures were labeled with FM4-64 for cell-shape determination.

In Fig. 10 the major stages analyzed by TIRF microscopy are represented in the form of three-dimensional reconstructions. In a normal cell, polymerized actin is most strongly enriched at the leading edges (Fig. 10 A). Latrunculin A treatment results in the destruction of actin complexes throughout the entire cell, and pearling occurs most abundantly near the substrate-attached cell surface (Fig. 10 B). The patches formed during the first phase of repolymerization extend ~2 μm from the bottom surface into the interior of the cell body. Some patches that appear separate when viewed close to the cell surface join to form a common cluster toward the interior (Fig. 10 C). At the turn from freely moving patches to the alignment of actin clusters at the cell border, the accumulation of actin is most pronounced in a zone close to the substrate surface (Fig. 10 D). Finally, the actin waves traveling on the substrate-attached cell surface extend 1–2 μm into the cytoplasmic space (Fig. 10 E). Together, these data show that the crucial events in actin reorganization occur close
to the plane of the substrate-attached cell surface that has been visualized at high-resolution by TIRF microscopy. The actin-enriched patches and waves associated with the substrate-attached cell surface grow further into the cytoplasmic space, but they end far below the upper cell membrane.

### DISCUSSION

**Actin repolymerization occurs separately from membrane protrusion**

The question addressed in this study is how leading-edge protrusion is initiated de novo after complete immobilization...
of a cell through actin depolymerization. Does actin polymerization restart from nucleation sites that are retained at the membrane during the depolymerization of actin, or can repolymerization begin anywhere in the cell and be subsequently coupled to membrane protrusion?

The data presented here provide evidence for the second mechanism. In cells recovering from the inhibition of actin polymerization by latrunculin A, the assembly of actin filaments is dissociated from its linkage to membrane protrusion. In that way the recovery of cell motility is dissected into two steps. First, the newly polymerized actin assembles into round clusters that move independently along the inner face of the plasma membrane. These clusters are transient structures; they may disassemble, fuse, or divide. Second, clusters collide at the cell border where their combined activity is able to advance the plasma membrane (Movie 2).

Actin reorganization as observed in Dictyostelium cells differs from that in Aplysia neuronal growth cones recovering from treatment with cytochalasin D, a plus-end capper of actin filaments. In the growth cones, clusters of short actin filaments survive the treatment. These clusters are either randomly dispersed or accumulated in a rim along the growth cone margin, and reorganization of the actin network occurs preferentially along this lamellar border (Forscher and Smith, 1988).

Actin depolymerization was complete in Dictyostelium cells under our conditions as judged by the absence of any actin-enriched structures detectable by TIRF or confocal scanning microscopy. Although we cannot rule out that short filaments are left that might function as nuclei of repolymerization, neither these hypothetical nuclei nor any other components initiate the repolymerization of actin at the cell
border where protrusion will eventually proceed. The turn from patch mobility to front protrusion is of particular interest as it demarcates a sudden switch in the control of polymerization.

Waves that travel in a plane close to the substrate-attached plasma membrane provide a second argument for the uncoupling of actin dynamics from membrane protrusion. These data imply that coordination of local activities into wave fronts does not require any machinery for actin nucleation that is located at the folded membrane of a leading edge. Nevertheless, a wave that travels against a cell border can cause the membrane to protrude, thus creating an activity that is typical of a leading edge (Fig. 9). The separation of actin polymerization from front protrusion during the initial phase of recovery opens a possibility of investigating the coupling of the two processes to each other. In this respect, it is of interest that cells can be arrested in the state of abundant wave formation by diluting latrunculin A from 5 μM down to 1 μM, rather than removing the drug completely.

The uncoupling of actin dynamics from force generation at the leading edge is most evident during the recovery from latrunculin A, but it is not restricted to these special conditions. Actin waves are observed in untreated cells (Bretschneider et al., 2002; Vicker, 2002), and they travel without a membrane fold in front of them, indicating that also in normally moving cells the coupling of wave dynamics to membrane protrusion is facultative (Bretschneider et al., 2004).

In untreated Dictyostelium cells, foci of dense actin assembly and traveling waves are inserted into a dynamic network of actin filaments, these dense structures being distinguished from the underlying network by a high content of the Arp2/3 complex (Bretschneider et al., 2004). With respect to their high Arp2/3 content, the foci and waves are comparable to the zone of ~1 μm in width that is associated with a leading edge (Fig. 3). Similarly, the patches and waves observed after treatment with latrunculin A are enriched in the Arp2/3 complex (unpublished data). The patches formed after latrunculin A treatment differ from the foci of untreated cells by their lateral mobility. The waves are much more profusely formed in cells recovering from latrunculin A. In following articles we will analyze the role played in the consecutive phases of actin repolymerization by activators of the Arp2/3 complex, such as members of the WASP and SCAR/WAVE families (Pollard and Borisy, 2003) or CARMIL (Jung et al., 2004).

In summary, there are two pieces of evidence indicating that actin dynamics in the cortex of Dictyostelium cells is only facultatively coupled to membrane protrusion. First, actin repolymerization starts with the formation of individually moving patches (Figs. 6 and 7 and Movies 2 and 3). These molecular complexes exist as transient structures that are capable of fusing and splitting into separate units. Secondly, actin waves travel on a planar membrane surface (Fig. 8 and Movie 4), until they meet the cell border where they provide force for pushing the membrane forward (Fig. 9). These data indicate that the wave front is capable of propagating autonomously, which means that its propagation does not rely on any machinery that might be embedded in the curved membrane of a leading edge. From these data three conclusions can be drawn:

1. The capability of actin polymerization can recover earlier than the molecular machinery that couples actin complexes to the membrane in a way that would empower a front to protrude.
2. A protruding front can be constructed from building blocks made of clustered actin filaments.
3. Waves of actin polymerization travel freely on the cytoplasmic face of a planar membrane until they are coupled to the cell border where they push the membrane forward in the form of a leading edge.

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

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